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Mechanisms of Action and Clinical Application of Macrolides as Immunomodulatory Medications

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INTRODUCTION

The term "macrolide" is used to describe drugs with a macrocyclic lactone ring of 12 or more elements (183). This class of compounds includes a variety of bioactive agents, including antibiotics, antifungal drugs, prokinetics, and immunosuppressants. The 14-, 15-, and 16-membered macrolides are a widely used family of antibiotics. They have excellent tissue penetration and antimicrobial activity, mainly against Gram-positive cocci and atypical pathogens (27). Macrolide concentrations are at least 10-fold higher in the epithelial lung fluid than in serum. Erythromycin A, a 14-membered macrolide, was isolated more than 50 years ago from cultures of *Streptomyces* and was the first macrolide introduced into clinical practice (183, 325). In this review, macrolide antibiotics are called "macrolides."

The nonantimicrobial properties of macrolides were suspected as far back as the 1960s (110), but their dramatic clinical effectiveness in treating diffuse panbronchiolitis (DPB) has served to extend their use to a number of chronic inflammatory diseases (71, 157, 202). DPB is a chronic debilitating disorder of unknown etiology primarily afflicting East Asians and resulting in refractory airway infection and life-threatening chronic respiratory failure. By helping to resolve unregulated and destructive inflammation, macrolides increased the 10-year survival rate from <40% in 1970 to 1979 to >90% after the widespread use of chronic erythromycin therapy (157). The characteristics of the clinical response to macrolide therapy are summarized as follows (71, 157, 202, 258): (i) it takes up to 3 months of therapy for macrolides to show a significant effect; (ii) doses that are much lower than the MIC (i.e., low-dose macrolide therapy) are effective; (iii) the effect is seen even when patients are infected with macrolide-resistant bacteria, such as *Pseudomonas*; (iv) clinical improvement is recognized even when bacteria persist in posttreatment sputum; and (v) these actions are seen only for treatment with 14- and 15-membered macrolides (e.g., erythromycin, clarithromycin, roxithromycin, and azithromycin), not with the 16-membered macrolide antibiotics (120). The effects of macrolides in patients with chronic inflammatory airway disease appear to be independent of antimicrobial properties. Immunomodulation, which differs from immunosuppression or anti-inflammation, is a nonlinear resetting of the inflammatory response by modifying or regulating one or more functions of the immune system (238). We use the term "immunomodulation" to describe the downregulation of a hyperimmunity or hyperinflammation without impairing the normal immune or inflammatory response to defend against infection.

This review summarizes what is known about the immunomodulatory activities of macrolides on the host and bacteria. Clinical applications are briefly discussed.

NONANTIMICROBIAL EFFECTS

Effects on Airway Secretion

The volume and biophysical properties of mucus or phlegm (airway secretions that would be called sputum if expectorated) play an important role in the regulation of mucociliary clearance. Hypersecretion is a feature of chronic airway inflammation and can cause airflow limitation, impairment of mucociliary transport, and recurrent respiratory infection. Macrolides can decrease mucus hypersecretion both *in vitro* (214) and *in vivo* (241, 290). Clarithromycin has been shown to improve the transportability of secretions in human subjects (241, 290). This mucoregulatory effect is seen even when hypersecretion is not induced by bacteria. Improved mucus transport may be associated with changes in the biophysical properties of secretions as well as with reduced inflammation.

Ion transport. Tamaoki and coworkers (289) studied the effects of macrolides on the airway bioelectric current measured in an Ussing chamber. Erythromycin and clarithromycin decreased short-circuit current (ISC), transepithelial potential difference (PD), and cell conductance in a dose-dependent manner, and these effects were not altered by a Na channel blocker but were abolished by a Cl channel blocker. Using a patch-clamp whole-cell technique, Ikeda and colleagues (104) showed that roxithromycin and erythromycin inhibit the acetylcholine-evoked Cl current in acinar cells isolated from the guinea pig nasal gland. This effect was thought to be due to inhibition of the Ca^{2+}-activated Cl channel. Likewise, erythromycin was shown to inhibit gamma interferon (IFN-γ)-induced outwardly rectifying chloride channel (ORCC) activation in cultured BEAS-2B cells (a human bronchial epithelial cell line) (76). The *in vivo* effects of macrolides on Cl channel activity were investigated in the rabbit tracheal mucosa. Intravenous administration of clarithromycin reduced the Cl diffusion potential difference in a dose-dependent fashion (288). These findings suggest that macrolides may reduce water and, possibly, mucin secretion through inhibition of the airway epithelial Cl channel.

However, there appears to be no significant effect of macrolides on chloride transport in persons with cystic fibrosis (CF). Barker and associates (23) investigated the effects of macrolides on airway epithelial ion transport in CF mice (both knockout and DeltaF508 homozygous mice) and human subjects. There was no effect of macrolides on PD across normal or CF nasal epithelium in either mice or humans, consistent with clinical reports (63).

There is a significant association between the increase of human calcium-activated chloride channel 1 (hCLCA1) mRNA and MUC5AC expression in asthmatics (321), and CLCA proteins may regulate mucin gene expression in humans (222). Modulation of this channel may be a promising treatment for mucus overproduction (204). Macrolides can affect ion channels by stabilizing Ca^{2+} homeostasis (151, 346).

In summary, although macrolides may have an effect on airway chloride ion transport, this seems to be an acute and dose-related effect and is probably not of clinical relevance.

Mucus secretion. Goswami et al. (83) studied the effect of erythromycin on mucus glycoconjugate secretion, using cultured human airway explants and also using an endometrial adenocarcinoma cell line. Following treatment with erythromycin, both cell types had decreased baseline mucus secretion. However, the mechanism of this effect was not explored.

Airway mucin is synthesized by epithelial goblet cells and by mucous cells of the submucosal glands. MUC5AC and MUC5B are the major gel-forming mucins in the human airway (281, 316, 317). Shimizu et al. (254) documented that erythromycin and clarithromycin inhibit tumor necrosis factor alpha (TNF-α)-induced mucus secretion in a dose- and time-dependent manner in NCI-H292 cells (a human muco-

epidermal cell line) and in nasal epithelial cells. This effect is accompanied by a reduced expression of MUC5AC mRNA. Immunohistochemistry showed that clarithromycin and roxithromycin inhibit mucus production induced by ovalbumin (OVA) instillation in OVA-sensitized rats or by lipopolysaccharide (LPS) exposure in rats (216, 254). Similar results were obtained after *Pseudomonas aeruginosa* infection in mice (125). In this investigation, clarithromycin inhibited mucus production and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in the lungs of *Pseudomonas aeruginosa*-infected mice.

The *P. aeruginosa* autoinducer *N*-3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL), a quorum sensing signal molecule, also stimulates the production of MUC5AC in NCI-H292 cells, through ERK1/2 and IκB phosphorylation (105). This induction was suppressed by azithromycin, and 3-oxo-C_{12}-HSL-induced MUC5AC production was blocked by the ERK pathway inhibitor PD98059. Similarly, stimulation of cells with LPS or transforming growth factor alpha (TGF-α) induces phosphorylation of IκBα (285). Macrolides inhibited LPS-induced MUC5AC gene expression and attenuated TGF-α-induced and LPS-induced phosphorylation of IκBα.

In summary, macrolides can inhibit mucus hypersecretion but do not appear to affect normal physiologic secretion. Macrolides are able to modulate mucin gene expression, most likely at the level of mitogen-activated protein kinase (MAPK) pathways or transcription factors, suggesting that this effect is part of a general modulation of immunity and inflammation.

Effects on Inflammation

Cytokine production. Cytokines and chemokines are key regulators of the inflammatory response, with both proinflammatory (e.g., TNF-α, granulocyte-macrophage colony-stimulating factor [GM-CSF], interleukin-1 [IL-1], IL-6, IL-8, and IFN-γ) and anti-inflammatory (e.g., IL-10) effects. Macrolides appear to decrease the production of proinflammatory cytokines that are detrimental to the host (48, 240).

In an earlier clinical study, erythromycin (600 mg daily for 3 months) reduced IL-8 and neutrophil elastase in bronchoalveolar lavage (BAL) fluid from subjects with chronic airway diseases, including those infected with *P. aeruginosa* (213). In subjects with DPB, treatment with erythromycin or roxithromycin for up to 24 months decreased IL-1β, IL-8, and neutrophils in BAL fluid (244). Other studies have shown that macrolides suppress proinflammatory cytokines in human airway washings and in serum (17, 133, 135, 198, 283).

IL-8 is one of the cysteine-X-cysteine (CXC) chemokines and is a potent neutrophil chemoattractant. Erythromycin inhibits release of IL-8, epithelial cell-derived neutrophil attractant (ENA-78), and macrophage inflammatory protein 1 (MIP-1) from macrophages and leukocytes (248). Clarithromycin can suppress LPS-induced IL-8 production in human peripheral monocytes and the human monocytic leukemia cell line THP-1 (140). Oishi and associates (213) reported that erythromycin inhibited IL-8 production from human neutrophils stimulated with formalin-killed *P. aeruginosa*. Erythromycin and clarithromycin also showed a concentration-dependent suppression of IL-8 release by eosinophils isolated from atopic subjects (149).

Physiological concentrations of erythromycin and clarithromycin inhibit IL-8 mRNA and protein in bronchial epithelial cells from healthy subjects and those with chronic inflammatory airway diseases (283). Erythromycin at a concentration of 10 μg/ml inhibited IL-6, IL-8, and soluble intercellular adhesion molecule 1 (sICAM-1) secretion from human bronchial epithelial cells (BEC) stimulated by endotoxin (135). Erythromycin decreased production of TNF-α and IL-6 in human whole blood stimulated with heat-killed *Streptococcus pneumoniae* (249). Similar effects have been observed using roxithromycin, which suppressed the production of IL-6, IL-8, and GM-CSF in the human epithelial cell line Bet-1A stimulated with IL-1α (133). Media conditioned with TNF-α-stimulated A549 human airway epithelial cells prolonged neutrophil survival in culture, an effect that was abolished by pretreatment of A549 cells with macrolides, which inhibited TNF-α-induced GM-CSF expression at both the protein and mRNA levels (333). While azithromycin stimulated TNF-α secretion from murine CF airway epithelial cells (75), azithromycin at 10 ng/ml in primary BEC from human lung transplant recipients inhibited release of IL-8 and GM-CSF after stimulation with LPS (201). This result may be due to the time of measurement, as it is characteristic of immunomodulation to both stimulate and suppress cytokine suppression, depending on the length of exposure.

As immunomodulatory drugs rather than immunosuppressive drugs, macrolides do not show simple time-dependent suppression of proinflammatory cytokines. Shinkai and associates (255) confirmed the multiphase response of IL-8 release from normal human BEC stimulated with LPS in the presence of macrolides. Addition of 10 μg/ml clarithromycin immediately decreased IL-8 production, then potentiated the LPS-evoked response (2.5-fold), and subsequently normalized IL-8 to the LPS-untreated control level. This effect was preceded by phosphorylation of ERK1/2, a p44/42 MAPK (255, 257). These findings are similar to those reported by Culić et al. for human neutrophils (49). The reason for this nonlinear response may be related to interaction with cross talk of intracellular signal transduction or oscillation-damping feedback (3, 315). ERK mediates normal human bronchial epithelial (NHBE) cell proliferation, and clarithromycin affects the transition from G_1 phase to S phase of the cell cycle and delays growth of NHBE cells (256). This effect is consistent with a previous *in vivo* study in which the oral administration of roxithromycin to healthy BALB/c mice increased levels of IL-1 and IL-2 activity for the initial 14 days, followed by decreases to the control level after 42 days of therapy (153). It has been proposed that macrolides first activate leukocytes and then suppress cytokine production in the presence of inflammatory priming (49). Acute neutrophil activation by a macrolide can facilitate the killing of microorganisms, while the suppression of chronic inflammation may limit airway damage (221). These studies suggest that macrolides initially increase the production of proinflammatory cytokines but that this then rapidly normalizes via immunomodulation.

Azithromycin slows the rate of lung function decline in CF patients. Cigana and colleagues (41) used three CF (IB3-1, 16HBE14o-AS3, and 2CFSMEo−) and two isogenic non-CF (C38 and 16HBE14o-S1) airway epithelial cell lines to investigate whether azithromycin reduced TNF-α mRNA and protein levels. Azithromycin reduced TNF-α mRNA levels and decreased TNF-α secretion, to approximately the levels in the

isogenic non-CF cells. NF-κB and specificity protein 1 (Sp1) DNA-binding activities were also significantly decreased after azithromycin treatment. Similar to these findings, azithromycin significantly reduced lung TNF-α levels, and this was associated with inhibition of neutrophil recruitment to the lung in a murine model stimulated with *Pseudomonas* beads (304).

In addition to effects on neutrophils, macrolides probably inhibit eosinophil recruitment. Macrolides inhibit secretion of the eosinophil-chemotactic cytokines RANTES and eotaxin (245). Erythromycin significantly decreased TNF-α-induced eotaxin mRNA as well as eotaxin release from human lung fibroblasts. Roxithromycin at 5 mg/kg of body weight inhibited formation of IL-5 by mouse spleen cells (152). These doses are comparable to those in humans, and it was speculated that oral administration of roxithromycin can inhibit the function of Th2-type lymphocytes.

Human studies demonstrated that macrolides can reverse the serum IFN-γ/IL-4 (Th1/Th2) ratio (229, 324). Roxithromycin dose dependently and significantly inhibited both IL-4 and IL-5 secretion by T cells prepared from both healthy and allergic rhinitis donors without affecting IL-2 and IFN-γ levels (15). However, there are also contradictory data suggesting a shift from Th1 to Th2 cytokine production after the use of macrolides. Park et al. (219) quantified changes in Th1 and Th2 cytokines in BAL fluid from patients with DPB after long-term treatment with erythromycin. After the treatment, IL-2 and IFN-γ levels in the BAL fluid were significantly decreased and the IL-4, IL-5, and IL-13 levels were significantly increased. In the ovalbumin-sensitized rat, a model of Th2 stimulation, BAL fluid and lung tissues were examined for mRNAs for cytokines (171). In these experiments, erythromycin did not decrease Th2-related cytokines or mRNAs. Clarithromycin and other macrolides were reported to inhibit Th1 cytokines, such as IL-2 and TNF-α, more markedly than Th2-type cytokines produced by mitogen-stimulated human T lymphocytes *in vitro* (197).

More recent studies have evaluated the effect of macrolides on T-cell regulation by dendritic cells (DCs). Azithromycin and clarithromycin significantly upregulated the expression of CD80, a costimulatory molecule for T-cell activation, from murine bone marrow-derived DCs (270). Azithromycin, but not clarithromycin, increased the production of IL-10, and clarithromycin, but not azithromycin, inhibited the production of IL-6 by DCs. Moreover, azithromycin increased IL-10 and clarithromycin significantly decreased IL-2 when naive splenic T cells were cocultured with DCs stimulated with LPS and exposed to macrolides. These data suggest that macrolides modulate the functions of DCs and that these effects may be different among macrolides.

In summary, there are clear and compelling data demonstrating that the 14- and 15-membered (but not the 16-membered) macrolide antibiotics can decrease the hypersecretion of proinflammatory cytokines and chemokines in cell culture, in animal models of disease, and in persons with chronic inflammatory pulmonary diseases. This effect appears to be nonlinear, and thus truly immunomodulatory, with an initial and short-lived increase in inflammation followed by a sustained decrease of cytokine production and secretion to normal, noninflamed levels, conceptualized as a "resetting" of the circuits. As discussed later, many of these effects appear to be mediated through inhibition of ERK1/2 or downstream transcription factors.

Adhesion molecule expression. Adhesion molecules are necessary for neutrophils and other inflammatory cells to migrate into the airway in response to inflammatory signals. Mac-1 (CD11b/CD18) expression on peripheral neutrophils from patients with DPB is higher than that on cells from healthy volunteers (159). Roxithromycin treatment caused a significant decrease in the expression of Mac-1, which was associated with decreased neutrophil numbers in BAL fluid. There were no changes in lymphocyte function-associated antigen 1 (LFA-1) expression (159). Downregulation of the integrin CD11b/CD18 has also been reported with erythromycin (173). Erythromycin inhibited LPS-induced neutrophil recruitment to the middle ear of rats, in part by downregulating L-selectin and Mac-1 expression on peripheral blood neutrophils (61).

Roxithromycin at therapeutic concentrations inhibited neutrophil adhesion to epithelial cells and decreased the expression of ICAM-1 on IFN-γ-treated epithelial cells (133). It was reported that soluble ICAM-1 derived from cultured epithelial cells was also reduced by erythromycin treatment (135). A subsequent study has shown that 14-membered macrolides directly inhibit vascular adhesion molecule 1 (VCAM-1) mRNA induction and leukocyte migration into the lung in a mouse model of bleomycin-induced lung injury (169).

In summary, macrolides have been shown to decrease stimulated expression of adhesion molecules, which may contribute to resolution of airway neutrophilic inflammation.

Inflammatory cells. (i) Chemotaxis. Neutrophilic inflammation is one of the key cellular features of DPB. In DPB, long-term erythromycin therapy significantly reduces the number of neutrophils and neutrophil chemotactic activity in BAL fluid (119, 211), and this is associated with a profound decrease in IL-8 (244). Macrolides also inhibit neutrophil migration after acute lung injury in animals, induced by intratracheal instillation of LPS (119), *Pseudomonas* beads (304), or bleomycin (20, 134).

However, not all studies have demonstrated inhibition of neutrophil chemotaxis. The concentration of macrolide needed *in vitro* to inhibit chemotaxis of peripheral blood neutrophils from healthy volunteers was much greater than therapeutic doses (293). Anderson (6) demonstrated that erythromycin increased neutrophil migration in response to a leukocyte attractant *in vitro*. Chemotaxis of neutrophils from 8 CF subjects was not affected by 4 weeks of oral erythromycin treatment (34).

It is likely that the effects of macrolides on neutrophil chemotaxis are due to decreased production of chemoattractants and decreased expression of adhesion molecules (97, 285).

(ii) Chemical mediators. Neutrophil elastase stimulates degranulation of mucin granules and release of mucin glycoprotein by goblet cells and submucosal glands. This protease also regulates the expression of IL-8, ICAM-1, and MUC5AC mRNAs (252, 317). Macrolides have been thought to have anti-elastolytic properties in the inflamed airway (98). Erythromycin can act as an alternate substrate inhibitor of neutrophil elastase, and flurythromycin can inactivate this enzyme *in vitro* (82). In asthmatics treated with clarithromycin, there was a decrease in sputum neutrophil elastase and matrix metalloproteinase 9 (MMP-9) (260).

Macrolides may modulate the lipoxygenase pathway of arachidonic acid metabolism. Leukotriene B_4 (LTB_4), a metabo-

lite of arachidonic acid, is an important chemotactic factor for neutrophils and is elevated in patients with chronic airway disease. Erythromycin and roxithromycin reduced LTB_4 in BAL fluid or epithelial lining fluid (ELF) in subjects with DPB. This was associated with decreased neutrophil numbers and neutrophil chemotactic activity (203, 212).

Endothelin-1, a potent bronchoconstrictor and vasoconstrictor, is also a mediator of airway inflammation (68). Bronchial smooth muscle cells have specific binding sites for endothelin-1, and BEC of asthmatic patients release large amounts of active endothelin-1 (314). Erythromycin and clarithromycin suppress endothelin-1 expression and release by human BEC, but this was not seen with josamysin, a 16-membered macrolide, or with tacrolimus, a very large macrolide (284).

Erythromycin dose dependently attenuates the contractile response of isolated human bronchial strips to electrical field stimulation, suggesting that macrolides may block bronchoconstriction by inhibiting neurotransmitter release or the cholinergic response in airway smooth muscle (286).

(iii) ROS. Reactive oxygen species (ROS) are recognized as mediators of cell and tissue injury (46). Macrolides are able to inhibit the production of ROS from neutrophils (161), and this effect is incubation time dependent (1, 166). Macrolide inhibition of stimulated neutrophil or eosinophil superoxide generation has been reported in many studies (6, 7, 47, 173, 225). It has been suggested that this is due in part to cell membrane stabilization. Macrolides attenuate the membrane-destabilizing effect of bioactive phospholipids, such as lysophosphatidylcholine, platelet-activating factor (PAF), and lyso-PAF, and this is accompanied by a dose-related inhibition of superoxide production (7). Interference with phospholipase D/phosphatidic acid phosphohydrolase may also decrease superoxide generation by phagocytes (1, 225).

Nitric oxide (NO) is a second messenger that is produced by NO synthase (NOS) early in the inflammatory response. Erythromycin stimulates endothelial NOS (eNOS) production by a protein kinase A-dependent mechanism (189). This is believed to inhibit leukocyte adhesion to endothelial cells. On the other hand, inducible NOS (iNOS) in pulmonary alveolar macrophages is suppressed by erythromycin (291). The same group reported that clarithromycin inhibited *in vitro* iNOS mRNA expression induced by endotoxin and IFN-γ (148).

(iv) Apoptosis. Neutrophil recruitment and activation are usually followed by neutrophil clearance and resolution of inflammation. In chronic inflammatory lung disease, neutrophils continue to be recruited to the airway, die by necrosis, and release their granule contents, increasing lung damage. Macrolides may induce apoptosis of activated neutrophils (12, 106). Erythromycin increased cyclic AMP (cAMP) in neutrophils *in vitro*, and it accelerated apoptosis at 24 h (12). Azithromycin has also been reported to promote apoptosis of neutrophils, but this effect was not seen in the presence of *Streptococcus pneumoniae* (146). However, in other studies, no direct effects of macrolides on neutrophil apoptosis and survival were observed, although shortening of neutrophil survival was mediated indirectly through inhibition of GM-CSF release from epithelial cells or IL-8 production in activated neutrophils (307, 333).

Roxithromycin induces apoptosis of anti-CD3-activated Jurkat T cells *in vitro* by enhancing Fas-Fas ligand and caspase-3 but not caspase-8 (118). Similarly, apoptosis of unstimulated peripheral lymphocytes from healthy subjects is induced by macrolides through the Fas-Fas ligand pathway (107). Clarithromycin and azithromycin can also increase the phagocytosis of apoptotic epithelial cells and neutrophils by alveolar macrophages (92, 331).

Although macrolides may promote neutrophil apoptosis as a mechanism to promote the resolution of inflammation, these data have been difficult to confirm *in vivo* and may be of limited clinical relevance.

(v) Cell differentiation. Macrolides promote differentiation of the human monocytoid cell line THP-1 into macrophages (271). Azithromycin may also alter the macrophage phenotype. Azithromycin shifted a mouse macrophage cell line (J774) from the classical activated phenotype (M1) to the alternatively activated phenotype (M2), reducing secretion of proinflammatory cytokines and increasing the anti-inflammatory cytokine IL-10 (200). A similar downregulation of inflammatory cytokines by azithromycin was reported for M1-polarized CF alveolar macrophages (186).

Airway epithelial cells. (i) Epithelial barrier. Data suggest that macrolides may help to stabilize the epithelial cell membrane. In the inflamed airway, phospholipases A_2 cleave arachidonic acid from the cell wall, and subsequent metabolism of arachidonic acid produces proinflammatory mediators. Feldman et al. have shown that macrolides can stabilize cell walls, protecting them against activated phospholipases (67). Furthermore, neutrophils potentiated the ciliary dysmotility and epithelial damage caused by proinflammatory phospholipids; this was ameliorated by pretreating the leukocytes with macrolides (67).

Junctional complexes between epithelial cells are a physical and chemical barrier. Inhalation of PAF causes ciliary dysmotility and disrupts the tight junction barrier in the rabbit trachea (205). Oral administration of roxithromycin significantly ameliorated PAF-induced ciliary dysfunction and the increase in epithelial permeability. Azithromycin, but not penicillin or erythromycin, increased the transepithelial electrical resistance (barrier) of human airway epithelial cells cultured on filter supports (16). In this model, azithromycin induced processing of the tight junction proteins claudin-1, claudin-4, occludin, and junctional adhesion molecule A.

Epithelial β-defensins are components of innate immunity in the airway. Erythromycin increased bactericidal activity of the airway surface liquid taken from cultured human primary tracheal cells, bronchial BEAS-2B cells, and pneumocyte II-like A549 cells (109). Erythromycin significantly increased the production of human β-defensin-1 and β-defensin-2 mRNA and protein. On the other hand, macrolides appeared to decrease β-defensin-2 levels, but not β-defensin-1 levels, in BAL fluid from DPB patients (90).

Thus, although macrolides appear to beneficially affect the epithelial barrier and ciliary function, this may be secondary to the decrease in airway inflammation.

(ii) CFTR expression. Cystic fibrosis transmembrane ion conductance regulator (CFTR) is a member of the ATP-binding cassette (ABC) transporter superfamily whose function is the transport of a wide variety of substrates. Multidrug-resistant (MDR) protein and a P-glycoprotein also belong to the ABC transporter family and share sequence homology. It was

reported that a CF patient who was treated with chemotherapy for fibrosarcoma had a dramatic improvement in lung function and that *P. aeruginosa* was no longer isolated from this patient (162). An increase in MDR mRNA in the patient's nasal epithelial cells was found, whereas no MDR mRNA was detected in a CF subject who had not received chemotherapy. It was speculated that the upregulated MDR protein complemented deficient CFTR function, leading to clinical improvement. Erythromycin is known to upregulate P-glycoprotein expression in epithelial cells (73), suggesting that this may be a mechanism by which macrolides improve pulmonary function in CF (4).

However, Equi and collaborators (63) demonstrated that neither nasal epithelial MDR nor CFTR mRNA levels in adult subjects with CF were changed after 2 weeks of azithromycin treatment, during which time there was a significant improvement in forced exhaled volume in the first second of exhalation (FEV_1). In addition, data show that neither clarithromycin nor azithromycin changes the nasal potential difference, sodium absorption, or chloride secretion (23), and azithromycin has no effect on MRP promoter transcriptional activity (42).

Although macrolides are clearly beneficial in persons with CF, as discussed below, the data do not support the hypothesis that this is due to either expression or function of the CFTR protein.

(iii) TLRs. The Toll-like receptors (TLRs) are an evolutionarily conserved family of receptors that function in innate immunity by recognizing some invariant regions in bacterial molecules. TLR2 recognizes peptidoglycan and bacterial lipoproteins, and TLR4 and TLR5 recognize LPS and flagellin, respectively. LPS can promote mucus secretion through TLR4, even in neutropenic animals (287). Epithelial TLR signaling stimulated by LPS or flagellin is decreased by macrolides, and this appears to be mediated through the MAPK–NF-κB pathway (255, 257).

Macrolides have been shown to affect TLR expression. Peripheral blood mononuclear cells stimulated with LPS have increased TLR4 mRNA levels. This was suppressed by clarithromycin and was accompanied by decreased LPS-induced IL-8 production (218). In contrast, Yasutomi et al. (337) demonstrated that erythromycin did not affect mRNA levels of TLR4 in monocyte-derived dendritic cells but that TLR2 mRNA was upregulated. In TLR2-transfected HeLa cells infected with *S. pneumoniae*, erythromycin did not influence the activation of TLR2 (196).

Because the macrolides are antimicrobial drugs and many of the innate immunity TLRs recognize specific bacterial products as an initial inflammatory response, it is tempting to speculate that macrolides may act in part by decreasing TLR expression or function. However, the limited data available do not support this as a primary mode of action for the macrolides' immunomodulatory properties.

Other cells. (i) Fibroblasts. Roxithromycin was shown to inhibit fibroblast proliferation in nasal polyps from patients treated for 1 month before polypectomy (210). Similar effects were reported in another study, along with inhibition of IL-8 levels in nasal lavage fluid, which was thought to drive polyp growth (330). Clarithromycin dose dependently reduced the expression of IL-8 and NF-κB in human adenoidal fibroblasts (279), and erythromycin significantly decreased TNF-α-induced eotaxin mRNA as well as eotaxin release from human lung fibroblasts (245). EM703, a novel derivative of erythromycin A (discussed below), inhibited fibroblast proliferation and collagen production in the murine lung fibroblast cell line MLg2908 treated with TGF-β (170).

Vascular endothelial growth factor (VEGF) plays a role in tumor growth as well as in inflammation of the respiratory tract by enhancing neovascularization and vasopermeability. Clarithromycin and roxithromycin have been reported to inhibit VEGF production from fibroblasts stimulated by hypoxia or TNF-α (182).

MMPs are secreted by a wide variety of cells, including fibroblasts, and are important in the migration of inflammatory cells through the basement membrane (60). Roxithromycin, but not josamycin, suppressed the production of MMP-2 and -9 in nasal polyp fibroblasts induced by TNF-α *in vitro*, accompanied by suppression of MMP mRNA expression through the inhibition of NF-κB and activator protein 1 (AP-1) activation (124). The inhibition of MMPs by macrolides may reduce the extracellular spread of inflammation.

Kohyama and coworkers (150) documented that clarithromycin, but not ampicillin, minocycline, or azithromycin, had a concentration-dependent inhibitory effect on migration of human fetal lung fibroblasts (HFL-1 cells) stimulated with thromboxane A_2, whereas there was no effect on HFL-1 cell-mediated gel contraction, another function of fibroblasts at the wound area.

Modulation of fibroblast function by macrolides appears to occur *in vivo*, perhaps as a result of generalized immunomodulation. Inhibition of fibroblast migration by macrolides may regulate the wound healing response following tissue injury.

(ii) Vascular endothelial cells. Angiogenesis, the proliferation and migration of endothelial cells resulting in the formation of new blood vessels, is associated with lesion formation in chronic inflammation as well as with progressive growth of solid tumors (69). Roxithromycin inhibited endothelial cell migration *in vitro* (339). The 14-membered macrolides appear to reduce tumor angiogenesis (340). Likewise, roxithromycin inhibits TNF-α-induced VEGF production, which is associated with suppression of NF-κB and AP-1 (217). The same group demonstrated an anticancer effect secondary to inhibition of angiogenesis in the mouse dorsal air sac model (10). These inhibitory effects on angiogenesis may be beneficial to prevent the neovascularization associated with airway remodeling seen in chronic inflammatory disorders such as CF and bronchiectasis.

The macrolide effects modulating cellular functions and related references are summarized in Table 1.

Effects on Cell Signaling

There have been so many different reported effects of macrolides on cell signaling pathways that attempts to put these actions together as one mechanism is difficult. In fact, all macrolides seem not to have similar ranges or degrees of activity. Proposed mechanisms and their influence on cell signaling are discussed below.

Intracellular calcium. Intracellular Ca^{2+} plays a fundamental role in signal transduction and regulates many enzymes, such as proteases, phospholipases, and endonucleases (44).

TABLE 1. Immunomodulatory effects of macrolides on cellular functions

Target cells and actions	Reference(s)
Airway epithelial cells	
Inhibition of:	
Chloride secretion	76, 104, 151, 288, 289, 346
Mucus secretion	83, 125, 216, 254
Adhesion molecules	133, 135, 169
Proinflammatory cytokines	41, 135, 255, 257, 283, 333
Inflammatory mediators	284, 286
Enhancement of:	
Tight junction or cell barrier	16, 67, 205
Defensin	109
Neutrophils	
Inhibition of:	
Chemotaxis	20, 119, 134, 304
Adhesion molecules	61, 159, 173, 198
Proinflammatory cytokines	49, 213, 248, 307
Elastase	82, 260
Reactive oxygen species	1, 6, 7, 161, 166 173, 225
Promotion of:	
Chemotaxis	6
Apoptosis	12, 106, 146
Eosinophils	
Inhibition of:	
Proinflammatory cytokines	149
Reactive oxygen species	47
Lymphocytes	
Inhibition of proinflammatory cytokines	15
Enhancement of:	
Apoptosis	107, 118
Regulation by dendritic cells	270
Monocytes	
Inhibition of proinflammatory cytokines	140, 197
Enhancement of differentiation	271
Macrophages	
Inhibition of:	
Proinflammatory cytokines	186, 200, 248
Inducible NO	148, 291
Fibroblasts	
Inhibition of:	
Proliferation	170, 210, 330
Collagen	103, 341
Matrix protease	124
Proinflammatory cytokines	182, 245, 279, 330
Vascular endothelial cells	
Inhibition of:	
Angiogenesis	10, 217, 339, 340
Adhesion molecules	169

Moreover, alteration of intracellular Ca^{2+} homeostasis is an early event in the development of irreversible cell injury (209). In airway epithelium, intracellular Ca^{2+} mobilization induced by proinflammatory mediators influences various cell functions, including ion transport and mucus secretion (43, 141). Experiments on epithelial cell lines suggest that Ca^{2+} signaling is involved in activation of the ERK1/2–NF-κB pathway and in ATP release as well as TLR-mediated responses by *P. aeruginosa* or flagellin (70, 184, 231, 277). Furthermore, intracellular Ca^{2+} agonists such as bradykinin and ATP can also increase cytokine expression and secretion in airway epithelia (199, 234, 235).

We and others (126, 127, 151, 289) have shown that macrolides inhibit purinoceptor-mediated intracellular Ca^{2+} oscillations and Ca^{2+} influx in primary cultured airway epithelial cells, thereby reducing Cl secretion, perhaps through suppression of Ca^{2+}-activated Cl channels. Similarly, Zhao et al. (346) have shown that erythromycin inhibits the ATP-mediated Ca^{2+} increase by suppressing Ca^{2+} influx from the extracellular space in A549 cells. Although molecular mechanisms of macrolide action on Ca^{2+} dynamics remain unclear, erythromycin does not affect verapamil-sensitive, voltage-dependent Ca^{2+} channels (151, 346). The effect of macrolides on the calcium response has been observed in other cells. Ca^{2+} influx into neutrophils and the oxidative burst were inhibited by erythromycin but not clarithromycin, suggesting that effects of macrolides on calcium mobilization are not completely homogenous (189). Recently, roxithromycin was shown to suppress histamine release and prostaglandin D_2 production from human β-defensin-2-stimulated mast cells, and this was accompanied by inhibition of the intracellular Ca^{2+} increase (130).

Calcium signaling after activation of apical G-protein-coupled receptors by proinflammatory mediators is amplified in CF airway epithelia (234, 235). Data suggest that the increased Ca^{2+} signal in CF cells may be due to the endoplasmic reticulum Ca^{2+} store increase or to inositol 1,4,5-triphosphate receptor (IP3R) dysfunction (9, 234). Macrolides may normalize Ca^{2+} responses and stabilize intracellular Ca^{2+} levels.

MAPKs. MAPKs form a signaling network that responds to diverse extracellular and intracellular stimuli, controlling a vast array of physiological processes. Three well-characterized subfamilies of MAPKs have been identified: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (116, 344). These MAPK signaling pathways interact and play an important role in the regulation of inflammatory cytokine gene expression and cell proliferation, differentiation, and apoptosis (93, 345). Activation of p38 MAPK can induce the production of inflammatory cytokines such as TNF-α, IL-1β, GM-CSF, and RANTES (87, 185). ERK1/2 and JNK can mediate proinflammatory cytokine levels (309), and MAPKs can regulate IL-8 promoter activity by NF-κB-dependent and -independent processes (168). ERK and other MAPK pathways are involved in the pathogenesis of hyperimmune reactions and inflammatory diseases (28, 174, 185) and appear to be major targets of macrolide immunomodulation.

ERK and p38 MAPK increase IL-8 expression in human bronchial cells (168, 185, 228). ERK can also activate NF-κB in human airway epithelial cells (28, 39). Shinkai and associates (255) focused on the ERK pathway and evaluated effects of macrolides on IL-8 and GM-CSF release from NHBE cells in culture. Clarithromycin exposure decreased ERK activity in the initial 30 to 90 min, the level was increased at 2 h to 3 days compared with that of the control, and it normalized to the unstimulated baseline level by 5 days. It was hypothesized that this was due to interactions of intracellular signal

transduction or negative feedback mechanisms (3, 315). These data are consistent with the well-defined time lag after macrolide administration to effective modulation of inflammation.

Inhibition of DNA-binding activity by macrolides may be due in part to suppression of upstream cell signaling. Clarithromycin increased IL-8 release at 24 h, an effect that was abolished by an ERK inhibitor but potentiated by a p38 MAPK inhibitor, suggesting that p38 MAPK may downregulate IL-8 release by NHBE cells (255). A similar increase in ERK activation was seen with flagellin-induced IL-8 secretion via the ERK pathway (257). Enhancement of the ERK pathway by the p38 MAPK inhibitor was postulated for a leukemia cell line (30), and thus cross talk between ERK1/2 and p38 MAPK may be involved in the immune response (181).

The effects of macrolides on MAPK signaling are not limited to cytokine production. Clarithromycin or an ERK inhibitor decreased MUC5AC gene expression and ERK1/2 phosphorylation in *P. aeruginosa*-infected mouse lung homogenates, suggesting that clarithromycin inhibits MUC5AC glycoprotein production through ERK inhibition (125). Azithromycin inhibited 3-oxo-C_{12}-HSL-induced MUC5AC production through inhibition of ERK phosphorylation in NCI-H292 cells (105). ERK1/2 activation was also inhibited in neutrophils treated with azithromycin (304). Erythromycin inhibited IL-1β-induced p38 MAPK phosphorylation in rheumatoid synovial cells *in vitro* (72).

The preponderance of published data strongly suggest that a primary immunomodulatory mechanism of action is through the macrolide effects on MAPK activation, in particular, via the ERK1/2 pathway. This makes an exceptionally compelling story, as it explains most of the observed immunomodulatory effects of these drugs and, to a great extent, predicts that MAPK-independent immune effects are less likely to occur as a primary effect. These effects are so reproducible that it has been suggested that modulation of ERK1/2 can be used as a drug screen for the immunomodulatory effects of novel macrolides.

Transcription factors. Transcription factors, including NF-κB and AP-1, regulate gene transcription in inflammatory responses (282, 285). These transcription factors bind upstream of promoters and regulate a number of actions, including generation of cytokines, chemical mediators, and oxidative metabolites. Aoki and Kao (11) investigated the effects of erythromycin at the level of transcriptional regulation of cytokine gene expression in Jurkat T cells. Erythromycin significantly inhibited NF-κB activation induced by phorbol 12-myristate 13-acetate (PMA) and calcium ionophore and suppressed IL-8 but not IL-2 expression. Erythromycin did not inhibit transcriptional activation of IL-2 and DNA-binding activity of nuclear factor of activated T cells (NFAT). This suggests the inhibition of IL-8, NF-κB, and DNA-binding activities by erythromycin. Similar findings have been reported for airway epithelial cell, fibroblast, monocyte, and leukemia cell lines (2, 55, 102, 140, 191).

Desaki and collaborators (55) reported that erythromycin and clarithromycin inhibited PMA-induced activation of NF-κB and AP-1 in the human bronchial epithelial cell line BET-1A, as well as the release of IL-8, whereas the macrolides showed no effect on the activation of cAMP-responsive element binding protein (CREB), suggesting that the effect on transcription regulation may be specific. Furthermore, erythromycin did not affect the phosphorylation of inhibitor of NF-κB (IκB), which is a crucial step for transactivation of NF-κB, indicating that erythromycin may act at the level of nuclear translocation of NF-κB or at the stage of DNA binding within nuclei (54). In a CF airway cell line, azithromycin inhibited NF-κB and Sp1 DNA-binding activities, reducing TNF-α secretion (41).

Macrolides also inhibit mRNA expression of mediators and cytokines such as IL-1, endothelin-1, iNOS, and MUC5AC (191, 216, 284, 291). The mechanism for this is likely to involve suppression of NF-κB and AP-1 (2, 11, 55, 140).

There are extensive data documenting the immunomodulatory effects of macrolides on transcription factors such as NF-κB. Interactions between MAPK signaling and transcription factor expression and function can explain most of the immunomodulatory effects observed. Figure 1 shows putative intracellular mechanisms by which macrolides can exert their immunomodulatory actions, and Fig. 2 gives a schematic presentation of potential immunomodulatory sites in the inflamed airway.

EFFECTS ON BACTERIA

DPB and CF are associated with *P. aeruginosa* infection, which causes progressive pulmonary damage. In a mouse model of endogenous *P. aeruginosa* bacteremia, low-dose erythromycin improved survival, from 20% for control mice to 80% for treated mice (89). A nonbactericidal effect on this organism has been postulated as one mode of action (96).

Inhibition of Adherence

Adherence of organisms to epithelium is essential for establishing a chronic bacterial infection. Baumann et al. studied the effect of roxithromycin by using a model of *P. aeruginosa* adherence to buccal epithelial cells from 11 CF children (25). Increased adherence was found in CF patients, and 3 months of azithromycin therapy decreased the adherence to normal control levels. Low-dose erythromycin also altered *P. aeruginosa* morphology and reduced adherence to human type IV basement membrane collagen *in vitro* (305). Yamasaki et al. (332) showed that exposure of *P. aeruginosa* to a sub-MIC level of erythromycin reduced the number of pili and, hence, adherence. Erythromycin suppresses the production of *P. aeruginosa* hemagglutinins, including the adhesins lectins as well as extracellular protease, hemolysin, and homoserine lactone autoinducers (266). Azithromycin at concentrations below the MIC inhibited flagellin expression and the motility of *P. aeruginosa* (132). One study using strain PAO1 and 13 strains (8 nonmucoid and 5 mucoid types) isolated from sputa of CF patients demonstrated that the decrease in adherence observed after azithromycin treatment is a strain-dependent event, although azithromycin at sub-MIC levels reduced bacterial adherence to bronchial mucins for many nonmucoid strains (36). The increased binding to CF cells may be related to mutant CFTR, in that cationic liposome-mediated CFTR gene transfer *ex vivo* significantly reduced adherence (52), but there was no effect on *P. aeruginosa* adherence to epithelial cells from CF patients after 2 weeks of azithromycin treatment (63).

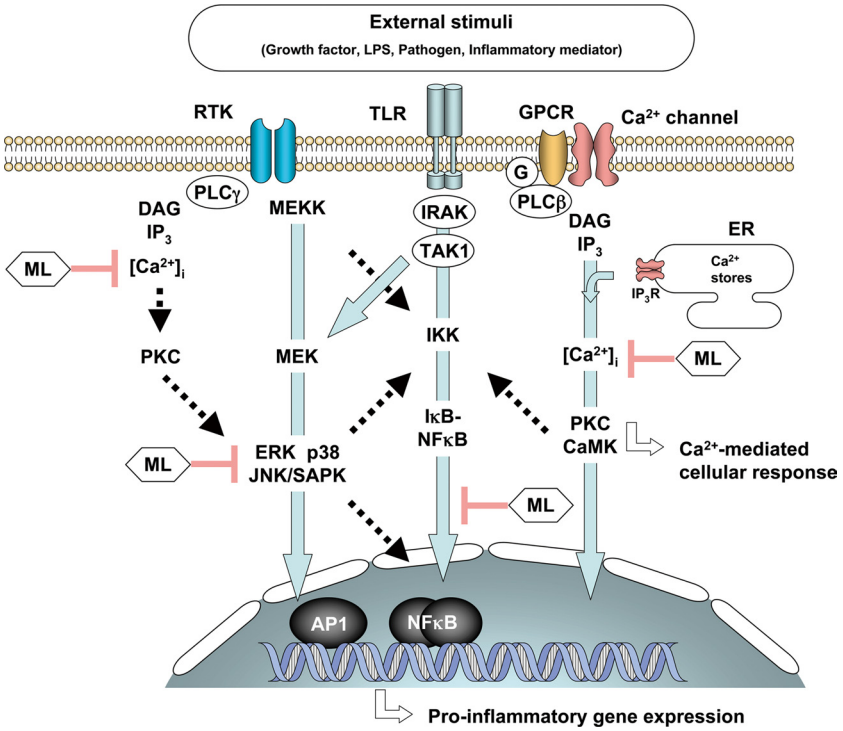

FIG. 1. Intracellular signal transduction pathways that have been proposed to be involved in macrolide immunomodulation. There are three major pathways that are influenced by macrolides. Receptor tyrosine kinases (RTKs) are receptors for many polypeptide growth factors and cytokines. RTKs such as epidermal growth factor receptor (EGFR) stimulate MEKK, and this activates the MAPK cascade. MAPK phosphorylates and activates transcription factors inducing proinflammatory genes (345). Macrolides inhibit MAPK activation, in particular, ERK1/2 activation. TLRs recognize bacterial molecules. For example, in response to LPS stimulation, TLR4 and adaptor molecules (not shown) activate the IRAK family and TAK1. TAK1 then stimulates two distinct pathways, the IKK complex and the MAPK pathway. The latter leads to the induction of AP-1, while the former activates NF-κB through the degradation of IκB proteins and the subsequent translocation of NF-κB (194). G-protein-coupled receptor (GPCR)- or RTK-mediated activation of phospholipase C (PLC) produces inositol triphosphate (IP_3). IP_3 is a ligand for the intracellular IP_3R channel of the endoplasmic reticulum's internal Ca^{2+} stores. Activation of PLC also leads to the production of diacylglycerol (DAG), which in turn activates protein kinase C (PKC). PKC and Ca^{2+}/calmodulin signaling are then activated (44). Macrolides inhibit intracellular Ca^{2+} increase. Abbreviations: AP-1, activator protein 1; CaMK, calmodulin kinase; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GFR, cytokine receptor/growth factor receptor; GPCR, G-protein-coupled receptor; IKK, IκB kinase; IP_3R, inositol triphosphate receptor; IRAK, IL-1 receptor-associated kinase; MEK, MAPK/ERK kinase; PKC, protein kinase C; TAK1, transforming growth factor-activated protein kinase 1; TLR, Toll-like receptor. Blue arrows, major pathways influenced by macrolides; dashed arrows, subpathways or cross-talk pathways; red lines with "ML," inhibition by macrolides; white bent arrow, cell response.

Inhibition of Virulence Factors

Tanaka and associates (292) showed *in vitro* that erythromycin inhibits the nasal epithelial damage caused by neutrophils in combination with filtrates of *P. aeruginosa*. However, preincubation of neutrophils alone with erythromycin was without effect, suggesting that erythromycin might reduce *P. aeruginosa*'s production of factors that damage epithelia or stimulate neutrophil-mediated cytotoxicity. At sub-MIC levels, macrolides inhibit the synthesis of exotoxin A, elastase, phospholipase C, DNase, lecithinase, gelatinase, lipase, and pyocyanin, all of which are virulence factors associated with *P. aeruginosa* infection (89, 143, 193, 195, 266). Tateda and coworkers (297) showed that a 48-hour exposure to macrolides at sub-MIC levels decreased the viability of both mucoid and nonmucoid *P. aeruginosa* strains. It was speculated that the time-dependent suppression of bacterial proteins, including GroEL, a major stress protein required for survival, might be lethal (298). Because of this, long-term macrolide therapy may shift the host-pathogen interaction from infection to a relatively benign colonization state. Pretreatment *P. aeruginosa* isolates from subjects randomized to receive azithromycin in a U.S. trial were analyzed, and the results suggested an *in vitro* effect of azithromycin, which decreased the production of phospholipase C by the isolates; this correlated with the pulmonary function (FEV_1) improvement seen with azithromycin therapy (208).

Inhibition of Biofilms

Chronic infection is a major cause of reactive inflammation and tissue destruction in DPB and CF. Strains of *P. aeruginosa* involved in these disorders develop a mucoid phenotype, synthesizing alginate, a component of bacterial biofilms, when environmental conditions are unfavorable for survival (144). The mucoid phenotype is associated with a change in morphology from flagellar motility to twitching motility, a loss of pili and flagella, and exopolysaccharide alginate production by guanosine diphospho-D-mannose dehydrogenase (GMD). Within mature

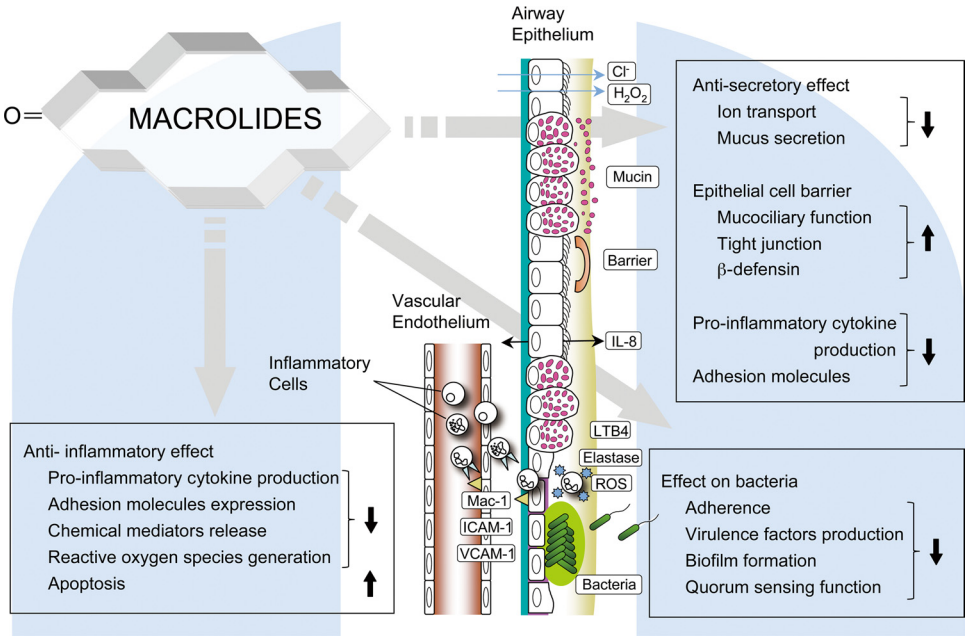

FIG. 2. Beneficial effects of macrolides in the inflamed airway. In a chronically inflamed airway, there is epithelial cell damage, infiltration of inflammatory cells, goblet cell hyperplasia, hypersecretion, mucociliary dysfunction, and recurrent airway infection. Macrolides have been reported to attenuate inflammation and cellular damage in a variety of ways, as represented in this diagram. Downward-facing arrows, inhibition; upward-facing arrows, enhancement.

biofilms, the bacteria are resistant to attack by antibacterial agents and phagocytic cells. Alginate can also act as an antigen and induce antigen-antibody reactions in the airways, producing circulating immune complexes in the host (145). Immune complex deposition in the airway, with resultant neutrophil chemotaxis, leads to lung damage (95). Sub-MIC levels of macrolides can inhibit alginate production (100, 336). Macrolides decreased the number of viable bacteria in a murine model of biofilm-associated respiratory *P. aeruginosa* infection (335). Decreased biofilm formation may be due to inhibition of the GMD production cycle (145, 188) or to prevention of pilus-dependent twitching motility (328). Long-term treatment with clarithromycin can decrease serum immune complexes in patients with DPB (144). Because macrolides can disrupt biofilms and revert bacterial morphology to a planktonic state, combining macrolides with antipseudomonal antibiotics may be more effective than using antibiotics alone for bacterial killing (145).

Inhibition of Quorum Sensing

Cell-cell communication among bacteria is mediated by chemical signaling molecules termed autoinducers, which increase in concentration as a function of cell density. Chemical communication involves producing, releasing, detecting, and responding to these small autoinducers, and this process is referred to as quorum sensing (187). In Gram-negative bacteria, including *P. aeruginosa*, the quorum sensing system comprises the genes encoding transcriptional activators (R genes) and autoinducer syntheses (I genes). The I genes (i.e., *lasI* and *rhlI*) are engaged in the biosynthesis of the corresponding autoinducer signal molecules, i.e., 3-oxo-C_{12}-HSL and *N*-butyryl-L-homoserine lactone (C_4-HSL), which regulate the production of virulence determinants and the formation of biofilms (295). Sputum samples from CF patients chronically infected with *P. aeruginosa* contain mRNA transcripts for the quorum sensing genes (268) and the autoinducers 3-oxo-C_{12}-HSL and C_4-HSL (261), indicating that quorum sensing signaling profiles consistent with a biofilm mode of growth are operative in CF isolates.

Azithromycin at a concentration of 2 μg/ml suppressed transcription of *lasI* by 80% and that of *rhlI* by 50% in *P. aeruginosa* strain PAO1 (294). Additionally, the production of 3-oxo-C_{12}-HSL and C_4-HSL was decreased to 6% and 28% of the control levels, respectively, suggesting that azithromycin interferes with the synthesis of autoinducers, leading to a reduction of virulence factor production. Azithromycin decreased bacterial loads, neutrophil infiltration, and lung pathology in *P. aeruginosa*-infected CF mice, in part by blocking quorum sensing (94). Similarly, erythromycin, clarithromycin, and roxithromycin, but not oleandomycin or josamycin, suppressed *lasI* gene expression (295).

The quorum sensing molecule 3-oxo-C_{12}-HSL is also a potent stimulator of host cells and can induce IL-8 production from human bronchial epithelial cells *in vitro* (56, 264) and produce inflammation *in vivo* (265). This molecule can induce macrophages and neutrophil apoptosis (296).

In summary, the effects of macrolides on biofilm disease include (i) inhibition of alginate production; (ii) suppression of the antibody reaction to alginate, thereby decreasing immune complex formation; and (iii) inhibition of *lasI* and *rhlI* expression and deactivation of the autoinducer 3-oxo-C_{12}-HSL in quorum sensing systems in *P. aeruginosa*. This activity may improve the resolution of the airway biofilm diseases CF and DPB (145).

TABLE 2. Inhibition of ERK1/2 and NF-κB pathways by macrolides

Pathway (references)	Response	Stimulator or cell type
ERK1/2 (105, 125, 255, 256, 257, 304)	Inhibition of mucus secretion, MUC5AC production, and MUC5AC gene expression	3-Oxo-C_{12}-HSL, *P. aeruginosa* infection, NCI-H292 cells
	Inhibition of proinflammatory cytokine secretion (IL-8, GM-CSF)	Flagellin, NHBE cells
	Inhibition of cell proliferation	NHBE cells
	Inhibition of chemotaxis	Neutrophils
NF-κB (11, 41, 54, 55, 102, 105, 124, 140, 191, 216, 279, 285)	Inhibition of mucus secretion, MUC5AC production, and MUC5AC gene expression	TNF-α, LPS, TGF-α, 3-oxo-C_{12}-HSL, NCI-H292 cells
	Inhibition of proinflammatory cytokine secretion (TNF-α, IL-1, IL-6, IL-8)	CF cells, 16HBE cells, BET-1A cells, T cells, monocytes, fibroblasts
	Inhibition of MMPs	Fibroblasts
	Inhibition of iNOS	Macrophages

SUMMARY OF MECHANISMS OF IMMUNOMODULATION

A wealth of supportive and detailed data support the observation that in mammalian cells, macrolides influence intracellular MAPK, especially ERK1/2, and the NF-κB pathway downstream of ERK. Because these pathways are involved in many cellular functions, including inflammatory cytokine production, cell proliferation, and mucin secretion, effects on ERK1/2 and NF-κB can explain most of the reported immunomodulatory effects of the macrolides (Table 2). The specific proteins or receptors targeted by macrolides that affect MAPK/NF-κB signaling have not been identified. The putative binding molecule(s) may have multiple mechanisms of action. Unpublished data suggest that portions of the macrolide molecule not involved in either antimicrobial actions or motilin receptor binding are responsible for immunomodulation. This information has been used to develop nonantimicrobial, immunomodulatory macrolides, as discussed below.

CLINICAL EFFECTS

Asthma

The immunomodulatory effects of macrolides were first reported for the treatment of patients with asthma in the 1960s, when troleandomycin (TAO), a 14-membered macrolide, was shown to decrease the amount of oral corticosteroid needed for asthma control in steroid-dependent asthmatics (110). This "steroid-sparing" effect was initially thought to be due to the action of TAO on corticosteroid metabolism via the cytochrome P450 system. TAO can reduce the clearance of methylprednisolone (276), but the clinical reduction in the dose of methylprednisolone achieved after TAO is far greater than would be anticipated from its effects on corticosteroid metabolism alone (342). A number of patients with steroid-dependent asthma were able to discontinue prednisolone entirely while on TAO (122, 237, 262, 318), suggesting that direct effects on the inflammatory response were involved. Similar properties were not seen with other antibiotics. In contrast, in a double-blind placebo-controlled study enrolling 75 adult asthmatics, there was no significant reduction in steroid use for 2 years, but the group receiving TAO had more side effects, such as reductions in bone density (207). Routine use of TAO for the therapy of asthma was largely limited by hepatic side effects (122, 207). A Cochrane review analyzed available data from 90 patients with steroid-dependent asthma in three randomized controlled studies of TAO (64). In this report, there was no treatment effect for TAO to reduce the steroid dose (standardized mean difference [SMD], −0.29; 95% confidence interval [95% CI], −0.75 to 0.17) and no significant improvement in lung function (SMD, 0.06; 95% CI, −0.8 to 0.9).

Gotfried and associates (84) evaluated add-on therapy of asthma, using clarithromycin administered at 500 mg twice daily, in a 6-week randomized controlled pilot study of 21 subjects with corticosteroid-dependent asthma. In the clarithromycin group, there were increases in forced vital capacity (FVC; $P = 0.04$) and FEV_1 ($P = 0.07$), as well as decreases in nocturnal dyspnea ($P = 0.05$) and chest discomfort ($P = 0.02$), compared with baseline values. In addition, subjects were able to reduce their prednisone dosage by a mean of 30% from baseline. Favorable effects of macrolide therapy were also observed in patients who were not taking oral corticosteroids. Miyatake et al. (192) evaluated the effects of 10 weeks of treatment with erythromycin (200 mg given three times daily) in a group of 11 atopic and 12 nonatopic asthmatics not receiving steroid therapy. Erythromycin decreased the degree of bronchial hyperresponsiveness in both groups, as measured by a significant increase in the provocative concentration required to produce a 20% fall in FEV_1 from the baseline (PC_{20}) in response to a histamine challenge. They also reported that there was no significant difference in serum theophylline concentrations before and after erythromycin treatment. A similar study was performed on children with asthma, using roxithromycin (253). The PC_{20} significantly increased with a dose of 150 mg roxithromycin daily after 4 and 8 weeks of treatment. No significant changes in serum theophylline concentrations and serum cortisol levels were observed during this study. Kamoi et al. (123) evaluated the effects of roxithromycin on the generation of free radicals by neutrophils and on bronchial hyperresponsiveness in 10 asthmatics over the course of a 3-month treatment and observed significant reductions in oxygen radicals and airway hyperreactivity compared with those in untreated controls. In a randomized controlled crossover clinical study, clarithromycin, given at 200 mg twice a day (b.i.d.), or placebo was administered for 8 weeks to 17 subjects with mild to moderate asthma (5). There were significant decreases in sputum eosinophils and eosinophilic cationic protein

as well as in bronchial hyperresponsiveness compared to those for the placebo group. These results were consistent with a study to evaluate the effects of clarithromycin on bronchial hyperresponsiveness (154). Subjects with stable asthma who were using budesonide at 400 mg daily were randomized to three study arms. Group A (22 subjects) received clarithromycin at 500 mg daily for 8 weeks, group B (20 subjects) received clarithromycin at 750 mg daily, and group C (21 subjects) received placebo. Bronchial hyperresponsiveness was quantified by measurement of the provocative dose required to produce a 20% fall in FEV_1 (PD_{20}) in response to methacholine provocation. Compared to the baseline, there was a significant increase in the median PD_{20} in groups A and B but not in the placebo group. The median values (interquartile ranges) for the three groups before and after treatment were as follows: group A, 0.3 (0.1 to 1) and 1.3 (0.6 to 2) mg ($P < 0.001$); group B, 0.4 (0.1 to 0.9) and 2 (2 to 2) mg ($P < 0.001$); and group C, 0.4 (0.1 to 0.9) and 0.3 (0.1 to 0.6) mg (not significant). The effectiveness of azithromycin was demonstrated in another study in which 11 subjects with mild asthma received 250 mg azithromycin orally twice weekly for 8 weeks and had significantly increased PC_{20} values in response to methacholine, without changes in FEV_1 (59). These macrolides had little or no effect on CYP3A4, one of the cytochrome P450 enzymes (224).

Asthma is a complex, multifactorial disease. Atypical organisms such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are thought by some to be associated with chronic asthma and asthma exacerbations (32, 155). Black and colleagues (31) conducted a randomized clinical trial of roxithromycin given at 150 mg twice daily for 6 weeks in 232 asthmatic subjects who were antibody positive for *C. pneumoniae*. There was no statistically significant change in the mean morning peak expiratory flow (PEF) or symptom score, but there was a significant improvement in evening PEF. These effects disappeared over a 6-month follow-up period after stopping the treatment. The prevalence of *M. pneumoniae* or *C. pneumoniae* in subjects with chronic asthma has been investigated by PCR, using BAL fluids (155). Clarithromycin given at 500 mg twice daily for 6 weeks improved the FEV_1 from 2.50 ± 0.16 to 2.69 ± 0.19 liters ($P = 0.05$) in PCR-positive subjects, but there was no significant effect in PCR-negative subjects. A Cochrane review updated in 2007, evaluating 7 studies including 416 subjects with chronic asthma, did not show a clinically significant benefit of using macrolides in the management of chronic asthma (236). This may suggest differences in disease duration and severity, the relatively short treatment duration in most studies, and the small number of subjects. A more recent clinical trial was performed with children who required medium to high doses of inhaled corticosteroids (ICS) with salmeterol to achieve asthma control (269). After a budesonide-stable period of 6 weeks, 55 children were randomized to receive once-nightly azithromycin, montelukast, or placebo plus the established controlling dose of budesonide (minimum of 400 mg twice daily) and salmeterol twice daily. The primary outcome was the time from randomization to loss of asthma control. There were no differences in time to loss of control (median for azithromycin, 8.4 weeks [95% confidence limit, 4.3 to 17.3 weeks]; median for montelukast, 13.9 weeks [95% confidence limit, 4.7 to 20.6 weeks]; and median for placebo, 19.1 weeks [95% confidence limit, >11.7 weeks]), but the study may not have been powered adequately.

In contrast, a placebo-controlled study to evaluate an 8-week course of azithromycin in 16 asthmatic children showed decreased bronchial hyperresponsiveness to hypertonic saline inhalation, and there was a reduction in airway neutrophil infiltration in the azithromycin-treated children but not in the placebo group (227). A randomized clinical trial with 45 adults with severely refractory asthma showed that 8 weeks of clarithromycin at 500 mg twice daily reduced concentrations of sputum IL-8, neutrophil elastase, and MMP-9 and improved clinical outcomes, including quality-of-life scores and wheezing frequencies, compared with those for the placebo group (260). There was no change in predicted FEV_1%, airway hyperresponsiveness to hypertonic saline, or the asthma control score. The authors found that the improvements in inflammation were most marked in those with refractory noneosinophilic asthma. Asthmatics who benefit from macrolide therapy appear to have cases of more-severe, steroid-resistant asthma, and this may be related to airway neutrophilic inflammation.

Non-CF Bronchiectasis

The effectiveness of macrolides in treating DPB has led to trials in other lung disorders characterized by productive cough, airway infection, and chronic inflammation. A randomized controlled trial of low-dose clarithromycin was reported for treatment of chronic inflammatory airway diseases, including bronchiectasis and chronic bronchitis (290). In the group that received clarithromycin at 100 mg twice daily for 8 weeks, sputum production decreased from 51 to 24 g/day and the percent solids composition increased from 2.4% to 3.0%, with no effect seen in the placebo group. Furthermore, the elasticity of the sputum increased, but its viscosity was unchanged. Similarly, in 11 patients with bronchiectasis, erythromycin given at 500 mg twice daily for 8 weeks decreased the 24-h sputum volume and improved pulmonary function compared with those of the placebo group (306). There was no change in sputum bacteria, leukocytes, IL-1α, IL-8, TNF-α, or LTB_4. Tagaya and coworkers reported reduced sputum volume with a short course of clarithromycin at 400 mg daily for 7 days (278). Thirty-eight percent of the subjects with chronic bronchitis or bronchiectasis had decreased sputum compared to the baseline volume; amoxicillin and cefaclor treatment had no effect on sputum volume.

A small open-label trial of azithromycin at 500 mg twice per week for 6 months in subjects with bronchiectasis also suggested a clinical benefit, as the patients had fewer exacerbations (50). In a randomized controlled trial, roxithromycin was given for 12 weeks to 13 children with airway hyperresponsiveness and bronchiectasis. The subjects taking roxithromycin had decreased sputum purulence and leukocyte counts, and the PC_{20} in response to methacholine increased significantly compared with that for the placebo group (147).

Low-dose roxithromycin decreased sputum IL-8, neutrophil elastase, and C5a and reduced neutrophil recruitment into the lung in subjects with bronchiectasis (203). BAL fluid IL-8 and neutrophil percentages, but not TNF-α or IL-10 percentages, decreased compared with those for controls in 17 children with

stable bronchiectasis after 3 months of clarithromycin given daily at 15 mg/kg (329).

COPD

Chronic obstructive pulmonary disease (COPD) is an epidemic disease predicted to become the third leading cause of death in the world by 2020. There are no pharmacological therapies today that will prevent disease progression or reduce mortality once COPD is established (24). Frequent exacerbations in COPD patients lead to a faster decline in lung function (57) and to increased airway inflammation (29). Many proinflammatory cytokines, chemokines, adhesion molecules, proteinases, and reactive oxygen species are involved in COPD pathogenesis (24).

The results of studies using macrolides to treat COPD have been mixed. Compared to placebo ($n = 36$), 3 months of oral clarithromycin given to subjects with stable COPD ($n = 31$) did not significantly improve health status or decrease sputum bacteria or the exacerbation rate (22). In contrast, Suzuki et al. (275) studied 109 Japanese subjects with COPD who were treated with low-dose erythromycin in an open-label study, and this was associated with decreased exacerbations and hospitalizations. These subjects were randomly assigned to prophylactic erythromycin therapy or no active treatment and were observed for 12 months. The frequency of upper respiratory tract infection was 76% for controls and 13% for the erythromycin group, and the exacerbation rate was 56% for the controls and 11% (6/55 patients) for the erythromycin group (relative risk [RR], 4.71; 95% CI, 1.53 to 14.5; $P < 0.007$). In an *in vitro* study by the same group, this effect was thought to be due to inhibiting human rhinovirus (RV) adherence by suppressing ICAM-1, an RV receptor, blocking RV RNA entry into the endosomes and decreasing proinflammatory cytokine production (274). Jang et al. (114) also showed that clarithromycin can decrease RV titers in infected A549 pulmonary epithelial cells. A retrospective multicenter analysis of 123 Japanese patients observed over a mean of 42.9 months indicated that macrolide therapy reduced the frequencies of COPD exacerbations and hospitalizations due to exacerbations (334). The frequency of exacerbations was 4.4% for the macrolide group ($n = 45$) and 15.4% for those not receiving chronic macrolide therapy ($P = 0.01$). Seemungal et al. (250) conducted a 12-month randomized clinical trial of erythromycin, given at 250 mg b.i.d. ($n = 53$), or placebo ($n = 56$) in subjects with moderate to severe COPD. There were 206 moderate to severe exacerbations, and 125 occurred in the placebo arm. The odds ratio for exacerbations for the erythromycin- compared with placebo-treated subjects was 0.65 (95% confidence interval, 0.48 to 0.86; $P = 0.003$), and subjects receiving erythromycin had shorter exacerbations.

Alveolar macrophages isolated from patients with COPD who had been given azithromycin had enhanced phagocytosis of apoptotic bronchial epithelial cells, and this was associated with increased expression of the mannose receptor (91).

Chronic Rhinosinusitis

Chronic low-dose macrolides were first used as immune modulators for chronic sinusitis in Japan (139, 142, 280), but this has also been studied in other countries (88, 241). In a 2-week randomized controlled study of clarithromycin given at 500 mg twice daily, there was a decreased nasal secretion volume (500 mg versus 28 mg; $P = 0.01$) and increased secretion mucociliary transportability (by 30%; $P = 0.005$) compared with those for controls. Clarithromycin also normalized the rheology and cohesivity of nasal mucus (88). Another study showed that clarithromycin treatment for 4 weeks increased spinnability (cohesivity) and decreased the ratio of viscosity to elasticity (tangent delta) of nasal mucus in 18 subjects with chronic sinusitis (233).

Hashiba and Baba (86) investigated the clinical efficacy of clarithromycin, given at 400 mg daily for 12 weeks, in 45 subjects with chronic sinusitis. Subjects taking clarithromycin had improved rhinoscopic findings and fewer symptoms of nasal obstruction, and the benefits correlated with the duration of therapy. There was a decrease in the size of nasal polyps associated with chronic sinusitis in 52% of patients after 8 weeks of roxithromycin treatment, given at 150 mg daily, but no correlation with allergy or eosinophilic infiltration (101). In another study, there was a decrease in nasal lavage fluid IL-8 for subjects with chronic rhinosinusitis whose nasal polyps were smaller after 12 weeks of clarithromycin treatment at 400 mg per day ($P < 0.005$) (330). It was speculated that IL-8 may drive the development of nasal polyps in some persons with rhinosinusitis. It has also been shown that macrolides can decrease IL-8 production by nasal epithelial cells *in vitro* (272).

In a prospective, single-center, open-label study, 25 subjects with stable chronic sinusitis and persistent maxillary sinus inflammation were treated with clarithromycin at 500 mg twice daily for 14 days (176). There were improvements in clinical signs and symptoms which lasted for up to 2 weeks after completing therapy, including significant decreases in edema scores and nasal biopsy specimen elastase, IL-6, IL-8, and TNF-α levels compared with those measured pretreatment.

Macrolide therapy also benefits patients with chronic persistent rhinosinusitis after sinus surgery. Cervin and colleagues (38) conducted a prospective open-label study of 17 subjects who did not respond to sinus surgery, antibiotics, and systemic steroids. Subjects were treated with erythromycin at 250 mg twice daily or with clarithromycin at 250 mg once daily. After 3 months, 12 of the 17 participants subjectively improved, and these responders were reassessed after 12 months of treatment. At 12 months, there were significant improvements in saccharin transit time (testing mucociliary clearance) ($P < 0.05$), nasal endoscopic scoring ($P < 0.01$), and symptoms of nasal congestion, sticky secretions, and runny nose by visual analog scale scoring ($P < 0.01$).

A randomized clinical trial was conducted with 64 subjects with chronic rhinosinusitis who were given 3 months of roxithromycin at 150 mg daily. There were significant improvements in SinoNasal Outcome Test 20 (SNOT20) scores, nasal endoscopy results, saccharin transit times, and IL-8 levels in lavage fluid compared with those for the placebo group ($P < 0.05$), and a correlation was noted between improved outcome measures and low initial IgE levels (319). This is consistent with the inhibitory effect of macrolides on neutrophilic inflammation (272, 330) and with studies suggesting that symptomatic improvement after macrolide therapy correlates with low

levels of IgE and with low eosinophil counts in peripheral blood, nasal smears, and the nasal mucosa (101, 273).

CF

CF is a genetic disease that is clinically and bacteriologically similar to DPB. Because of these common features, an open pilot study of erythromycin given for 4 weeks to CF patients was performed and showed that sputum IL-8 concentrations for 6 subjects with CF were decreased (65). Jaffe and colleagues (113) then evaluated long-term (mean duration, 0.6 years) azithromycin therapy in 7 children with CF and infection with *P. aeruginosa*. These children had median increases in FVC of 11.3% and in FEV_1 of 11% ($P < 0.03$).

Wolter and colleagues (326) reported the results of a prospective placebo-controlled study of 60 adults with CF who were given daily azithromycin, at 250 mg, or placebo for 3 months. FEV_1 was maintained in the azithromycin group, while there was a significant decline in the placebo group. Systemic inflammation, as measured by C-reactive protein levels, decreased only in the azithromycin group. Quality of life improved over time in patients on azithromycin and remained unchanged in those on placebo. Similarly, Equi et al. (62) conducted a placebo-controlled crossover trial with 41 children who received either azithromycin or placebo for 6 months. Twenty-two subjects were homozygous for the ΔF508 mutation. After 2 months of washout, the treatments were crossed over. Equi et al. found a 5.4% improvement in FEV_1 with azithromycin, but there was no effect on either bacterial density or sputum inflammatory markers. It was suggested that children homozygous for the ΔF508 mutation did better than the others. A larger, multicenter study also demonstrated significant lung function improvements and fewer intravenous antibiotic courses for subjects with CF who were on azithromycin (243). A total of 185 adults and children who had CF and were infected with *P. aeruginosa* were randomly assigned to receive placebo or 250 mg (for body weights of <40 kg) or 500 mg (for body weights of ≥40 kg) of azithromycin 3 days a week for 168 days. The predicted FEV_1% increased 4.4% in the azithromycin group but declined 1.8% in the placebo group (mean difference, 6.2%; $P = 0.001$). Subjects taking azithromycin had fewer exacerbations than those in the placebo group (hazard ratio [HR], 0.65; 95% CI, 0.44 to 0.95; $P = 0.03$), and they weighed an average of 0.7 kg more at the end of the study (95% CI, 0.1 to 1.4 kg; $P = 0.02$ compared with placebo group). A meta-analysis of 4 studies with 296 participants concluded that there is a small but significant treatment effect for azithromycin to improve pulmonary function in persons with CF (267).

In a 1-year randomized controlled study, 82 young CF patients (≥6 years old; mean age, 11.0 years) were randomized to receive azithromycin at 250 or 500 mg, depending on body weight, or oral placebo three times a week for 12 months (45). The relative changes in predicted FEV_1% at the end of the study did not differ significantly between the two groups, but the number of pulmonary exacerbations, the time to the first pulmonary exacerbation, and the number of additional courses of antibiotics were significantly reduced in the azithromycin group. Similarly, maintenance azithromycin therapy given over 3 years was assessed retrospectively in 100 patients with CF (303). The predicted FEV_1% improved in the first year after initiation of azithromycin but decreased during the second and third years. Another long-term observational cohort study of 45 adult CF patients with chronic *P. aeruginosa* infection demonstrated a significant decrease in the rate of decline in lung function (85). The negative annual median slope of decline of pretreatment FEV_1 was changed from −4.1% of the predicted value to a positive annual slope, with an increase of +0.8%, after 12 months of treatment with 250 mg daily azithromycin, corresponding to a difference of +4.9% ($P < 0.001$).

CF lung inflammation may represent a primary event due to the genetic defect. CF airway epithelial cells have a spontaneously increased production of IL-8 and TNF-α and an enhanced inflammatory response to bacteria (37, 41). Furthermore, CF airway cells produce inflammatory peptides even in the absence of infection (302), and they respond to *P. aeruginosa* with a greater-than-normal inflammatory response (247). In the F508del-CF mouse, there is spontaneous and exaggerated LPS-induced macrophage infiltration in the airways, but both spontaneous and induced inflammatory responses are attenuated following azithromycin treatment (163). Azithromycin downregulates proinflammatory cytokine expression in M1-polarized CF alveolar macrophages and wild-type alveolar macrophages (186). However, it is not clear whether this hyperinflammatory process is the direct or indirect result of the CFTR defect (175, 239). The level of phosphorylated ERK1/2, but not p38 MAPK, is markedly elevated in CF bronchial cells and further supports the hypothesis that CFTR, the intracellular Ca^{2+} response, and NF-κB activation of CF airway epithelial cells are interdependent (112, 175, 277).

DPB

The initial clinical trials of macrolides as immunomodulatory medications for the therapy of chronic inflammatory airway diseases were driven by the dramatic effects reported for use of these drugs to treat DPB. DPB is a progressive inflammatory airway disease, primarily reported from Japan, Korea, and Taiwan, which is characterized by a chronic cough, copious sputum expectoration, dyspnea, and chronic sinusitis (156). The airways of persons with DPB are infected by *Haemophilus influenzae*, *S. pneumoniae*, and eventually mucoid *P. aeruginosa*. The bacterial exacerbations lead to airflow obstruction, respiratory failure, and cor pulmonale. Symptoms usually appear in the fourth or fifth decade of life in nonsmokers, and the 10-year survival rate was <15% for patients infected with *P. aeruginosa* (71). Low-dose erythromycin was first used to treat DPB in 1982. Work by Kudoh and colleagues (158) demonstrated that low-dose erythromycin therapy significantly improves the long-term survival of DPB patients. Many subsequent trials have shown the effectiveness of erythromycin for the treatment of DPB (71, 99, 144, 202, 258). Erythromycin therapy improves symptoms, pulmonary function, and hypoxemia and increased the 10-year survival rate to >90% (156). The benefit has been demonstrated with other 14- and 15-membered macrolides as well (120, 258), but josamycin, a 16-membered macrolide, has been ineffective (19). A long-term study by Kadota et al. (120) reported the effectiveness of clarithromycin at 200 mg/day for 4 years in patients with DPB. Pulmonary function and oxygenation improved significantly

within 3 months of starting therapy, and the effects persisted for the full 4 years.

Neutrophil influx into airways and the production of oxidants and proteolytic enzymes play a crucial role in the pathogenesis of DPB (98). In DPB, macrolides decrease neutrophil chemotactic activity in BAL fluid. Erythromycin therapy decreased the number of neutrophils and neutrophil-derived elastolytic-like activity in BAL fluid (98). There were decreased numbers of neutrophils and neutrophil chemotactic activity in BAL fluid, not only for patients following erythromycin treatment but also for animal models (119, 211). Several studies have shown a reduction in BAL fluid levels of IL-8 and IL-1β for subjects with DPB following macrolide treatment (17, 198, 283). Additionally, there is decreased adhesion molecule macrophage activating complex 1 (Mac-1) in peripheral blood neutrophils after macrolide therapy (198). Macrolides suppress $CD8^+$ $CD11b^-$ cytotoxic T cells in BAL fluid from subjects with DPB (131). Macrolide suppression of proinflammatory cytokine production may be the principal mechanism of action in DPB (71, 183, 283).

The working group of the Diffuse Lung Disease Committee of the Ministry of Health and Welfare of Japan prepared clinical guidelines for macrolide therapy for DPB in 2000. The guidelines suggest that a macrolide be started as soon as the diagnosis of DPB is made, and erythromycin at 400 or 600 mg daily is recommended. If this treatment provides insufficient clinical benefits after 6 months of therapy or if undesirable adverse effects are associated with this drug, clarithromycin at 200 or 400 mg daily or roxithromycin at 150 or 300 mg daily is then recommended (206).

Influenza and Sepsis

Systemic infectious diseases such as severe influenza and sepsis can produce a severe systemic inflammatory response syndrome (SIRS). The parenchyma injury is associated with overexpression of cytokines and inflammatory mediators and with upregulation of adhesion molecules in vascular beds (40, 310, 320). Modulation of SIRS has been proposed on the basis of experimental and clinical evidence (79, 220, 230). In murine influenza virus-induced pneumonia, erythromycin dose dependently improves survival, and this is associated with inhibition of inflammatory cell responses and attenuation of NO production in the lung (246). Other studies demonstrated that clarithromycin suppresses inflammatory cytokines such as TNF-α (138) but increases IL-12 and immunoglobulin A in airway fluids (308), reducing mortality in treated mice. Azithromycin can significantly improve survival in mice infected with influenza virus and superinfected with *S. pneumoniae* (129). Improved survival appears to be mediated by decreased inflammation, manifested as fewer inflammatory cells and proinflammatory cytokines in the lungs. Clarithromycin also inhibits virus production from influenza A virus-infected host cells *in vitro* (190).

European and North American guidelines recommend a combination therapy consisting of a β-lactam and a macrolide for hospitalized patients with community-acquired pneumonia (CAP) (178, 327). However, there are no randomized controlled trials comparing a single β-lactam with the same drug supplemented with a macrolide, and these recommendations are largely supported by observational studies of selected populations which have shown lower mortality rates for patients treated with β-lactam–macrolide combination therapy than those for patients treated with β-lactam monotherapy (21, 35, 74, 180, 301). However, other studies failed to show a benefit of macrolide-containing combinations (58, 223). The synergy between β-lactams and macrolides against *S. pneumoniae* is not documented *in vitro* (172) or *in vivo* (115), although macrolides inhibit the production of the pneumococcal toxin, pneumolysin (8). Oral azithromycin decreased the rate of lethality in a septic shock model in mice challenged with intraperitoneal or intravenous LPS, accompanied by a reduced serum level of TNF-α, without suppressing neutrophil tissue infiltration (111). Therefore, the reason for the beneficial effect appears to be unrelated to β-lactam resistance.

In humans, the cytokine profile for 23 subjects with CAP treated with clarithromycin at 500 mg twice daily for 7 days was compared with that for subjects treated with amoxicillin at 1 g three times daily for 7 days. In the clarithromycin group, there was a decrease in the proinflammatory cytokine IL-6, an increase in the anti-inflammatory cytokine IL-10, and an increase of IFN-γ (53). At therapeutic concentrations, clarithromycin inhibited IL-8 release from human monocytes stimulated with bacterial extracts of *Escherichia coli* or *P. aeruginosa* (140).

Intravenous clarithromycin in the therapeutic range improved survival in a murine model of sepsis caused by susceptible *E. coli*, multidrug-resistant *P. aeruginosa*, or pandrug-resistant *Klebsiella pneumoniae*, and this was associated with decreased serum TNF-α and reactive oxygen species (78, 80, 81). These pathogens were not susceptible to clarithromycin *in vitro*, and the effects were observed even if clarithromycin was administered after the animals developed sepsis-associated pulmonary edema. TNF-α secretion by monocytes isolated from clarithromycin-treated animals was also decreased *ex vivo* (26).

A randomized, controlled, multicenter trial was designed to evaluate the efficacy of clarithromycin in patients with sepsis and ventilator-associated pneumonia (VAP) (79). Clarithromycin was administered intravenously at a dose of 1 g once daily for 3 consecutive days in 100 subjects, and another 100 subjects were treated with placebo. Other antimicrobials for treating VAP were selected by the attending physicians. Although 28-day mortality was not affected, clarithromycin shortened the median time for resolution of VAP and time to extubation among the 141 survivors compared with those for placebo (10.0 days versus 15.5 days [$P = 0.011$] and 16.0 days versus 22.5 days [$P = 0.049$], respectively). Fourteen subjects died of a cause other than sepsis, and 45 died of sepsis. The odds ratio for death due to a sepsis-related cause among clarithromycin-treated patients was reduced compared with that for placebo-treated patients (3.78 versus 19.00; $P = 0.043$). A pathogen was identified in 70% of cases, and the eradication rates were similar in both groups.

Restrepo and associates (232) found a survival benefit of macrolide therapy in 30- and 90-day mortality rates for patients with sepsis after pneumonia. In this retrospective cohort study, 104 (43.9%) of 237 subjects with severe sepsis received macrolides. Overall, 30- and 90-day mortality rates were lower for subjects who received macrolides than for the others (11% versus 29% [$P = 0.001$] and 12% versus 34% [$P < 0.001$], respectively). The multivariable analysis revealed that the use of macrolides was associated with decreased mortality at 30

days (HR, 0.3; 95% CI, 0.2 to 0.7) and at 90 days (HR, 0.3; 95% CI, 0.2 to 0.6) in patients with severe sepsis and in patients with macrolide-resistant pathogens (HR, 0.1; 95% CI, 0.02 to 0.5).

Posttransplantation BOS

Bronchiolitis obliterans syndrome (BOS) is the leading cause of death after lung transplantation. An open-label pilot study demonstrated that 6 subjects with BOS who were treated with azithromycin at 250 mg three times a week had improved FEV_1, with a mean increase of 17% ($P \leq 0.05$) after an average of 13.7 weeks (77). Four subsequent clinical trials of BOS showed significant improvements in FEV_1 with azithromycin treatment (136, 312, 313, 338), while one showed no demonstrable effect (259). The most recent of these studies treated 14 subjects with azithromycin at 250 mg daily for 5 days and then three times weekly for 3 months (313). There were 6 responders to azithromycin (with an FEV_1 increase of >10%), and the mean FEV_1 increased from 2.36 to 2.67 liters ($P = 0.007$). Azithromycin reduced BAL fluid neutrophils and IL-8 mRNA. The authors concluded that in subjects with >15% neutrophils in their BAL fluid, there was a positive predictive value of 85% for a significant FEV_1 response to azithromycin. Finally, pulmonary IL-17-induced IL-8 production is thought to be important in BOS, and azithromycin has been shown to inhibit IL-17 through its effect on MAPK signaling (311).

SAFETY OF LONG-TERM MACROLIDE THERAPY

Macrolide Resistance

The antimicrobial activity of macrolides is based on inhibition of bacterial protein biosynthesis after binding of the macrolide, selectively and reversibly, to the 50S ribosomal subunit. More precisely, macrolides prevent peptide bond formation by interacting with 23S rRNA, a major component of the 50S subunit and an important part of the peptidyltransferase center (179). The main binding sites lie within domain V in the 23S rRNA. In addition, it is postulated that macrolides cause dissociation of the peptidyl-tRNA during translocation by blocking the path through which nascent peptide chains exit the ribosome, thus interfering with elongation of the polypeptide chain (300).

There is a concern that the long-term administration of macrolides can promote antimicrobial resistance. Malhotra-Kumar and coworkers (177) conducted a randomized, double-blind, placebo-controlled study, enrolling a total of 224 healthy volunteers: 74 were treated with azithromycin, 74 with clarithromycin, and 76 with placebo. Oral streptococci were used as model organisms to evaluate the development of resistance over 180 days. Both macrolides significantly increased the proportion of macrolide-resistant streptococci, with resistance peaking at day 4 in the azithromycin group (mean increase of 53.4% versus placebo; $P < 0.001$) and at day 8 in the clarithromycin group (50.0% increase versus placebo; $P < 0.001$). For CF patients taking maintenance azithromycin therapy, marked increases in *Staphylococcus aureus* and *Haemophilus* spp. resistant to macrolides have been reported (226, 303). Staphylococcal resistance to macrolides went from 83% in the first year to 97% in the second year and, finally, to 100% in the third year after starting therapy (303). The long-term use of azithromycin in CF patients also leads to macrolide resistance in the commensal viridans group streptococci, as assessed by molecular characterization (299). Since the incidence of nontuberculous mycobacterial (NTM) pulmonary infections is also increasing among CF patients, it is feared that azithromycin therapy may induce selection or acquisition of macrolide resistance in NTM (167, 215).

There are three principal mechanisms responsible for acquired macrolide resistance (299, 343). The first is due to ribosomal target modification, which is mediated by methylases encoded by the *erm*(B) gene. The methylation of the adenine at position 2058 of domain V prevents macrolides from binding to the ribosome, resulting in high-level resistance. The second is an active drug efflux mechanism, which is determined by the presence of the membrane-bound efflux protein, encoded by the *mef*(A) gene. This mechanism confers low-level macrolide resistance. The third is a mutation in the 23S rRNA or ribosomal proteins L4 and L22, leading to conformational changes at the binding site of the macrolides and to a large reduction in effectiveness. The PROTEKT study is a global longitudinal surveillance study of the distribution of mechanisms for macrolide resistance to *Streptococcus pneumoniae* (66). Year 5 PROTEKT data (2003–2004) showed that the rate of erythromycin resistance in *S. pneumoniae* isolates from patients with community-acquired respiratory tract infections was 37.2%. The overall distributions of the resistance mechanisms *erm*(B) and *mef*(A) were 55.0% and 30.6%, respectively, with a combination of both mechanisms accounting for 12.0% of cases of resistance. Ribosomal mutations accounted for a small minority of cases (2.0%). Long-term use of macrolides for common conditions such as asthma and COPD should be examined comprehensively on the basis of the clinical evidence, and there may be a benefit to developing non-antimicrobial macrolides that retain immunomodulatory properties without inducing resistance (see "EM703").

Long-Term Macrolide Safety

Kadota and colleagues reported on the safety and effectiveness of once-daily clarithromycin given at a low dose for 4 years to 10 patients with DPB (120). They found that pulmonary function improved in most of these patients within 6 months, and often the improvement was seen within 2 to 3 months. There were also improvements in oxygen saturation at rest and in quality of life within 6 months in nine of these patients. These improvements persisted for at least 4 years, with no reported side effects. The same authors later reported on long-term macrolide antibiotic therapy in the treatment of chronic small-airway disease that clinically mimicked DPB but had a very different histology. Unlike the 80 to 90% response to long-term erythromycin therapy reported for DPB, pulmonary function, total cell counts, and neutrophil percentages in bronchoalveolar lavage fluid did not improve in these patients (121). However, similar to the case for patients with DPB, there were no reported side effects seen with long-term use. In a similar study, Phaff and colleagues from the Netherlands reported on the safety and effectiveness of long-term azithromycin therapy in patients with CF (226). In their CF center, 156 patients were monitored during the study period of Janu-

TABLE 3. Clinical application of macrolides

Disease	Recommendation for routine use	Expected efficacy	Outstanding issues or comments
Asthma	Discouraged (few patients)	Steroid-sparing effect	Insufficient evidence, small study population
Bronchiectasis	Partially recommended	Decreased sputum, reduced exacerbations	Few data, small number of patients, short duration
COPD	Discouraged	Reduced exacerbations	Insufficient evidence, small study population
Chronic rhinosinusitis	Partially recommended (for noneosinophilic cases)	Reduced nasal mucus, improved quality of life	Study design, study population
Cystic fibrosis	Recommended	Reduced exacerbations, less deterioration in pulmonary functions	Short duration
DPB	Recommended	Improved prognosis, potential cure	First-line therapy
Influenza/sepsis	Discouraged	Improved prognosis	Insufficient evidence
Posttransplantation BOS	Discouraged	Improved prognosis	Insufficient evidence

ary 1999 to March 2004, and by October 2003, 50 patients were taking azithromycin three times a week at a low dose. As the duration of treatment increased, sputum cultures of patients who were using azithromycin were positive for either *Staphylococcus* or *Haemophilus* significantly less often than cultures of patients not on azithromycin, although the percentage of resistant cultures in those that were culture positive dramatically increased. Macrolide resistance increased in both those taking azithromycin and those not using the antibiotic.

It has been reported that the concurrent use of CYP3A inhibitors and erythromycin can increase the risk of cardiac death. Postmarketing data surveillance from Japan indicates that although 10 tons of erythromycin are used annually for low-dose, long-term immunomodulatory treatment, there have been only 4 cases of torsade de pointes due to erythromycin among an estimated 34,000 patients per year treated with erythromycin over the last 10 years. Furthermore, these four patients received erythromycin without concomitant treatment with CYP3A inhibitors, and they recovered after discontinuing erythromycin treatment (18).

Thus, it appears that the long-term and low-dose use of macrolides can increase the development of resistance in Gram-positive organisms, apparently without compromising the effectiveness of the medications or increasing the risk of cardiovascular death. The development of nonantimicrobial macrolides that possess immunomodulatory properties would be expected to decrease the risk of macrolide resistance developing in both patients and community isolates. Guidance for chronic use of macrolide therapy is summarized in Table 3.

OTHER RELATED DRUGS

Ketolides

Ketolides are a class of semisynthetic agents derived from erythromycin A by replacement of the cladinose at C-3 with a keto group. Ketolides bind to ribosomes with a higher affinity than that of macrolides, and they potently inhibit protein synthesis by interacting with the peptidyltransferase site of the bacterial 50S ribosomal subunit (343).

Telithromycin was the first member of this class, and it has been reported to have immunomodulatory properties similar to those of the macrolides. In monocytes from eight volunteers, telithromycin inhibited LPS-induced IL-1α and TNF-α, but not IL-1β, IL-6, or IL-10 (14). In a septic mouse model, telithromycin downregulated production of TNF-α and IL-1β and improved the rates of survival after a lethal dose of *E. coli* LPS was given (165). Acute airway inflammation in mice after airway nebulization of LPS was attenuated by telithromycin pretreatment, and this was associated with decreased neutrophilia and reduced levels of protein, nitrite, MIP-2, and TNF-α in the BAL fluid (164). *In vitro*, telithromycin inhibited the production of MIP-2 and TNF-α in response to LPS stimulation in the macrophage cell line RAW 264.7, and it decreased the production of MIP-2 by murine lung epithelial (MLE-12) cells cultured with supernatants of LPS-stimulated RAW 264.7 macrophages. NF-κB activation was inhibited and apoptosis was increased in both cell lines by use of telithromycin (164). Telithromycin, as well as azithromycin and clarithromycin, can inhibit the increased production of MUC5AC mRNA and protein in NCI-H292 cells stimulated with LPS (108). However, telithromycin did not inhibit MUC5AC production induced by human neutrophil peptide-1 (HNP-1), while azithromycin and clarithromycin did inhibit MUC5AC.

Johnston et al. (117) evaluated the effects of telithromycin in 278 patients with acute exacerbations of asthma in a double-blind, randomized, placebo-controlled study. Telithromycin given at 800 mg daily for 10 days decreased asthma symptoms significantly compared with placebo, although there was no significant treatment effect on the morning peak expiratory flow. There was no relationship between the response to asthma treatment and *C. pneumoniae* or *M. pneumoniae* infection, as ascertained by serologic analysis, PCR, and culture.

Quinolones

Antimicrobial activity of fluoroquinolones is due to interference with DNA replication by inhibiting bacterial DNA gyrase and topoisomerase IV (51). Although eukaryotic cells do not contain these enzymes, fluoroquinolones accumulating intracellularly could potentially affect topoisomerase type II enzymes, the mammalian counterparts, thereby having the potential to damage mammalian cells (263).

Fluoroquinolones were also proposed as immunomodulators and have been compared with macrolides (51, 220). Fluoroquinolones are able to modify transepithelial Cl secretion, phagocyte functions, T-cell responses, and cytokine production (128, 160, 242). Moxifloxacin and ciprofloxacin dose dependently decreased IFN-γ and IL-4 expression by T cells *in vitro*

(324). Therapeutic concentrations of moxifloxacin significantly inhibited IL-1α and TNF-α secretions from human monocytes stimulated with LPS (13). In mice injected with a lethal dose of LPS, ciprofloxacin and trovafloxacin protected mice from death and decreased TNF-α and IL-6 levels in serum (137). Likewise, moxifloxacin inhibited IL-8 and TNF-α, preventing neutrophilic pneumonitis in immunosuppressed mice (251). Furthermore, moxifloxacin has been shown to suppress ERK1/2 activation and NF-κB nuclear translocation in human monocytes and a lung respiratory cell line (322, 323). Blau et al. (33) showed that moxifloxacin inhibited proinflammatory cytokines and MAPK activation, more potently than ciprofloxacin or azithromycin did, in TNF-α- or IL-β-treated CF airway cells. In differentiated human bronchial cells cultured at an air-liquid interface, moxifloxacin as well as azithromycin was able to inhibit TNF-α-induced IL-8 secretion (347). These data may be of relevance for the use of fluoroquinolones in the treatment of respiratory tract infections in patients with chronic airway inflammatory diseases.

Interestingly, immunomodulatory properties seem to be limited to members of the quinolone family with a cyclopropyl moiety at position N-1 of the quinolone ring (e.g., ciprofloxacin, sparfloxacin, and moxifloxacin) (51). The basic mechanisms for these effects of quinolones have not been evaluated. Further studies, including studies of a quinolone-topoisomerase II interaction with an effect on transcription factors, are required (51).

EM703

EM703 is a novel 12-membered-ring macrolide derivative of erythromycin which was developed by the Kitasato Institute for Life Sciences of Kitasato University in Japan (170, 271). EM703 has neither antimicrobial nor gastrointestinal motilin-binding effects. Similar to erythromycin, EM703 can inhibit PMA-mediated IL-8 release and NF-κB activation in BET-1A cells, a human bronchial epithelial cell line (54). EM703 can inhibit proliferation and collagen production of murine and human lung fibroblasts by interfering with TGF-β/Smad signaling (103, 341). The relationship between these effects and the three-dimensional structure of macrolides is under investigation.

CONCLUSIONS

Macrolides have many diverse biological activities and an ability to modulate inflammation. These properties have led to their use for treatment of DPB, asthma, bronchiectasis, rhinosinusitis, and CF. These actions seem to be polymodal and exerted at different levels of cellular signaling, but among these, modulation of ERK1/2 and transcription factors is prominent, consistent, and clearly unrelated to antimicrobial properties. Macrolides (and quinolones) accumulate within cells, suggesting that they may associate with receptors or carriers responsible for the regulation of immune cell activities.

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